DB=PGPB,USPT; PLUR=YES; OP=ADJ

L6 (18kd$ or '18 kd$') and (cd40L or cd40 adj ligand or cd154 or gp39)	40	L6
L5 (18kd$ or '18 kd$') same (cd40L or cd40 adj ligand or cd154 or gp39)	4	L5
L4 (18) same (cd40L or cd40 adj ligand or cd154 or gp39)	728	L4
L3 (18kda or '18 kd$') same (cd40L or cd40 adj ligand or cd154 or gp39)	4	L3
L2 L1 and (cd40L or cd40 adj ligand or cd154 or gp39)	0	L2
L1 lazarus.in.		

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L6: Entry 7 of 40 File: PGPB Jan 8, 2004

DOCUMENT-IDENTIFIER: US 20040006006 A9
TITLE: CD40-Ligand lacking native-pattern glycosylation

Summary of Invention Paragraph:
[0002] The present invention relates to CD40-Ligand (CD40-L). More specifically, the present invention relates to CD40-L molecules lacking native-pattern glycosylation.

Summary of Invention Paragraph:
[0009] Prior to the present invention, a ligand for CD40 was unknown. Accordingly, there is a need in the art to identify and characterize a CD40 ligand (CD40-L).

Detail Description Paragraph:
[0062] CD40 can be expressed, for example, by hematopoietic cells and ovarian cancer cells. Gallagher et al. (2002), Mol. Pathol. 55(2): 110-20. Epidermal growth factor receptor (EGFR) can also be expressed by ovarian cancer cells. Nicholson et al. (2001), Eur. J. Cancer 37(Suppl. 4): S9-15. Ligation of CD40 by adding to ovarian cancer cells a soluble version of CD40 ligand (CD40L), which is a CD40 agonist, leads to growth inhibition of these cells. Gallagher et al., supra. Similar effects of CD40 agonists have been observed in some other cancer cells. However, in normal B cells, a CD40 agonist provides an anti-apoptotic and proliferative stimulus, indicating that responses to CD40 agonists vary. Gallagher et al., supra.

Detail Description Paragraph:
[0064] CD40 agonists can include: polypeptides comprising all or part of the CD40 ligand (CD40L) or substantially similar polypeptides that can serve as agonists of CD40; part or all of an antibody or a substantially similar polypeptide that can specifically bind to CD40 and serve as an agonist of CD40, such as those described in U.S. Pat. Nos. 5,801,227, 5,677,165, or 5,874,082, among others; part or all of a polypeptide selected for CD40 binding in vitro or a substantially similar polypeptide that can serve as an agonist to CD40; CD40 antibodies, agonists, and binding proteins described in U.S. Pat. Nos. 5,801,227 and 5,674,492; and small molecules that can serve as CD40 agonists. In vitro selection schemes to obtain binding proteins are described in e.g. He and Taussig ((1997), Nucleic Acids. Res. 25(24): 5132-5134), Hanes and Pluckthun ((1997), Proc. Natl. Acad. Sci. 94: 4937-4942), Roberts and Szostak ((1997), Proc. Natl. Acad. Sci. 94: 12297-12302), Lohse and Wright ((2001), Curr. Opin. Drug Discov. Devel. 4(2): 198-204), Kurz et al. ((2000), Nucleic Acids Res. 28(18): E83), Liu et al. ((2000), Methods Enzymol. 318: 268-93), Nemoto et al. ((1997), FEBS Lett. 414(2): 405-08), U.S. Pat. No. 6,261,804, WO 00/32823, WO 00/34784, Parmley and Smith ((1989), Adv. Exp. Med. Biol. 251: 215-218), Luzzago et al. ((1995), Biotechnol. Annu. Rev. 1: 149-83), and Lu et al. ((1995), Biotechnology (NY) 13(4): 366-372). The sequences of CD40 and CD40L are known in the art. See Stamenkovic et al. (1989), EMBO J. 8: 1403-10; Spriggs et al. (1992), J. Exp. Med. 176: 1543-50. Methods for making antibodies are also known in the art. A variety of standard assays have been described for assessing whether a particular molecule can agonize CD40. Several assays for apoptosis, which CD40L prevents in osteoclasfs, are described in WO 01/16180 and in Gallagher et al., supra. Assays for cell proliferation, such as cell counting and optical density measurements, are well known in the art. Assays for expression of specific genes (as described in Gallagher et al. supra) can also be indicative of agonism of CD40.

Detail Description Paragraph:

[0110] It is also possible to utilize an affinity column comprising CD40 ligand binding domain to affinity-purify expressed CD40-L polypeptides. CD40-L polypeptides can be removed from an affinity column in a high salt elution buffer and then dialyzed into a lower salt buffer for use.

Detail Description Paragraph:
[0116] This example describes construction of a CD40/Fc DNA construct to express a soluble CD40/Fc fusion protein for use in detecting cDNA clones encoding a CD40 ligand. The cDNA sequence of the extracellular region or ligand binding domain of complete CD40 human receptor sequence was obtained using polymerase chain reaction (PCR) techniques, and is based upon the sequence published in Stamenkovic et al., supra. A CD40 plasmid (CDM8) was used as a template for PCR amplification. CDM8 is described in Stamenkovic et al. and was obtained from the authors. A PCR technique (Sarki et al., Science 239:487, 1988) was employed using 5' (upstream) and 3' (downstream) oligonucleotide primers to amplify the DNA sequences encoding CD40 extracellular ligand binding domain. Upstream oligonucleotide primer 5'-CCGTCGACCACCATGGTTCGTCTGCC-3' (SEQ ID NO:5) introduces a Sal 1 site upstream from an initiator methionine of CD40 and a downstream oligonucleotide primer 5'-ACAAGATCTGGGCTCTACGTATCTCAGCCGATCC- TGGGGAC-3' (SEQ ID NO:7) that inserts amino acids Tyr Val Glu Pro Arg (SEQ ID NO:8) after amino acid 193 of CD40. Glu and Pro are the first two amino acids of a hinge region of human IgG1, and are followed by a Bgl II restriction site that was used to fuse the extracellular domain of CD40 to the remained of human IgG1 Fc region.

Detail Description Paragraph:
[0159] These data indicate that the interaction of CD40 with its ligand is the principal molecular interaction responsible for T cell contact dependent induction of B cell growth and differentiation to both antigen-specific antibody production and polyclonal Ig secretion. As such, these data suggest that antagonists of this interaction, by soluble CD40, CD40/Fc fusion protein and possibly soluble CD40-L (monomeric), will significantly interfere with development of antibody responses. Therefore clinical situations where CD40, CD40/Fc fusion proteins and soluble CD40-L are suitable include allergy, lupus, rheumatoid arthritis, insulin dependent diabetes mellitus, and any other diseases where autoimmune antibody or antigen/antibody complexes are responsible for clinical pathology of the disease. Moreover, membrane-bound CD40-L or oligomeric soluble CD40-L will be useful to stimulate B cell proliferation and antibody production. As such, these forms of CD40-L are most useful for vaccine adjuvants and as a stimulating agent for mAb secretion from hybridoma cells.

Detail Description Paragraph:
[0179] A PCR technique using 5' (upstream) and 3' (downstream) oligonucleotide primers was used to amplify the DNA sequences encoding the CD40-L truncated extracellular domain from a cloning vector containing murine CD40-L. The upstream oligonucleotide primer (ATATGAATTCGACTACAAAGATGACGATGATAAACCTCAAATTGCAGCACACGTT; SEQ ID NO:18) appended an EcoRI site and the Flag coding sequence upstream from CD40 nucleotide 355. The downstream oligonucleotide primer (CCTTCGCGGCCGCGTTCAGAGTTT GAGTAAGCCAA, SEQ ID NO: 19) introduced a Not 1 site downstream of the authentic termination codon of the CD40L.

Detail Description Paragraph:
[0182] A similar CD40-L construct was made without an amino terminal Flag.RTM. sequence. This construct utilized an existing Bam HI site at nucleotide 351 in the CD40-L sequence and the downstream PCR oligonucleotide primer described above (SEQ ID NO:19). After amplification of the CD40 sequence with a 5' upstream oligonucleotide homologous to CD40-L nucleotides 324-346, and the downstream primer which introduced a Not I site, the PCR product was cut with Bam HI and Not I and ligated into pAcGP67A cut with Bam HI and NotI. This construct was cotransfected into SF21 cells along with viral DNA as previously described, and recombinant virus was plaque purified, expanded and used to infect insect cells to produce serum free

conditioned supernatants. CD40-L was detectable in these crude supernatants by both CD40Fc receptor binding assay and by detection of an 18 Kd band on a Coomassie Blue-Blue-stained PAGE. Similar copnstructs were also prepared for human CD40-L.

Detail Description Paragraph:
[0183] This example describes purification of trimeric murine CD40L from supernatant fluid from transfected CHO cells. A CHO cell line expressing muCD40LT was maintained in suspension in spinner-flask cultures. For production, the cells were centrifuged and resuspended into a controlled 3 liter bioreactor in serum-free medium. Oxygen, agitation and pH were controlled for at 40% dissolved O.sub.2 (relative to air saturation), 150 RPM and 7.2, respectively. The culture was harvested after nine days. A total volume of approximately 160 ml of supernatant fluid from the bioreactor was dialyzed overnight at 4.degree. C. against 4 L of 20 mM Tris pH 7.5 buffer containing 150 mM NaCl, and then adjusted to 1M (N).sub.2SO.sub.4 by the addition of solid (NH.sub.4).sub.2SO.sub.4. Dialysis accomplished the removal of low-molecular weight contaminants; other techniques will also be useful for this purpose, for example, constant volume diafiltration.

Detail Description Paragraph:
[0184] The dialyzed supernatant was initially purified by hydrophobic interaction chromatography. The supernatant was applied to a 1.6.times.13 cm (26 ml) Phenyl Sepharose.RTM. CL-4B column (Pharmacia, Uppsala, Sweden) previously equilibrated with 10 mM Tris pH 8.0/1M (NH.sub.4).sub.2SO.sub.4 (Buffer A). The column was washed with 60 mL Buffer A, and bound proteins were eluted at 2 mL/min with a decreasing (NH.sub.4).sub.2SO.sub.4 gradient using Buffer A and 10 mM Tris pH 8.0 (Buffer B). The gradient conditions were 0 to 60% Buffer B in 20 ml, hold at 60% Buffer B for 60 ml, 60 to 100% Buffer B in 20 ml, and hold at 100% Buffer B for 60 ml. A total of 53 3 ml fractions were collected during the elution process. The elution of protein was monitored by absorbance at 280 nm. The presence of active trimeric CD40L was determined by an ELISA as described in Example 16. A peak of activity eluted in fractions 8-20. In a subsequent purification run, highsub and lowsub Phenyl Sepharose 6 Fast Flow.RTM.P (Pharmacia, Uppsala, Sweden) were used for the hydrophobic interaction step; the highsub Phenyl Sepharose.RTM. column was found to give equivalent results to those obtained with Phenyl Sepharose.RTM. CL-4B.

Detail Description Paragraph:
[0186] Fractions from the peak of activity from the SUPERDEX 200.RTM. column were pooled, concentrated as described above to approximately 2.0 ml, diluted 1:2 in 20 mM Bis Tris Propane pH 6.5/10% glycerol (v/v), and further purified by anion exchange chromatography. The concentrated pooled material was applied at 1 mL/min to a MONO Q.RTM. column (Pharmacia, 0.5.times.5 cm) equilibrated with 20 mM Bis Tris Propane pH 6.5/10% glycerol (v/v) (Buffer A). The column was washed with 16 mL Buffer A and eluted with a salt gradient using Buffer A and 20 mM Bis Tris Propane pH 6.5/500 mM NaCl/10% glycerol (v/v) (Buffer B). The column elution conditions were 0 to 60% Buffer B in 20 ml, 60 to 100% Buffer B in 1.0 ml, and hold at 100% Buffer B for 10 ml. A total of 30 1 ml fractions were collected during the elution process. Activity and A.sub.280 were monitored as described previously. A peak of activity eluted in fractions 15-23. The fractions were evaluated by SDS-PAGE and silver stain as described above. Fractions 20-22 were estimated to contain about 80% trimeric murine CD40L, and were pooled. In a subsequent run, a HIGH PERFORMANCE Q.RTM. resin (Pharmacia, Uppsala, Sweden) was used and found to give equivalent results. Table 9 below summarizes the results of the procedure used to purify trimeric murine CD40L.

Detail Description Paragraph:
[0190] A second experiment was carried out using different lots of reagents and varying the concentrations of the CD40-L. A significant difference between the control mice and the mice treated with CD40 ligand was not observed at day 7, however, CD40-L did enhance the day 14 response. Additional experiments to address

the use of CD40-L will include an analysis of different antigens as well as the use of different adjuvants and delivery systems.

Detail Description Paragraph:
[0202] This example illustrates preparation of a number of muteins of a CD40 ligand/zipper domain fusion protein. Mutations for constructs to be expressed in yeast (mutants 14, 18, 32, 41, 43, 10PP and 18PP) were generated by PCR misincorporation (Mulrad et al Yeast 8:79, 1992), and selected based on an apparent increase in secretion as improved secretion mutants.

Detail Description Paragraph:
[0204] Mutant 18PP had only a single mutation in the molecule, which was sufficient to affect secretion in yeast. Mutant 41 had two mutations, both of which were in the isoleucine residues of the zipper domain. The mutations in the zipper improve secretion from yeast without apparent effect on activity. Mutant 194.W was expressed in yeast cells and purified either by a combination of ion exchange chromatography steps (194.W (c)) or by affinity chromatography (194.W (a)) using a monoclonal antibody that binds the oligomerizing zipper moiety. oligomerizing zipper moiety. The yeast-expressed mutant (194.W) exhibited greater affinity for CD40 in a biosensor assay performed substantially as described in Example 22, and exhibited greater biological activity than wild type CD40 ligand/zipper domain fusion protein (WT) expressed in yeast, in a B cell proliferation assay. These results are shown in Table 14.

Detail Description Table CWU:
9TABLE 9 Purification of Trimeric Murine CD40-L Volume Total # Total Protein Specific Step (ml) Binding Units (mg) Activity * 1. Supernatant fluid 160 5.2 .times. 10.sup.6 280 1.9 .times. 10.sup.4 2. Dialyzed 169 6.4 .times. 10.sup.6 106 6.0 .times. 10.sup.4 Supernatant 3. Phenyl Sepharose 37 2.3 .times. 10.sup.6 8.0 2.9 .times. 10.sup.5 pool 4. Superdex 200 24 2.3 .times. 10.sup.6 1.4 1.6 .times. 10.sup.5 pool 5. MONO Q pool 7 6.4 .times. 10.sup.5 0.91 7.0 .times. 10.sup.5 * Specific activity is defined as the number of binding units of CD40-L per mg protein. One binding unit of CD40L is defined as 0.5 ng of purified CD40-L, as determined in a quantitative, enzyme-based binding assay. Protein concentration was determined using the BCA Protein Assay Reagent (Pierce); bovine serum albumen was used as the standard.

Detail Description Table CWU:
12TABLE 13 Mutations present in the CD40 ligand/zipper domain fusion protein Zipper Mutant Domain No: Mutation.sup.a CD40L Domain Mutations.sup.b Activity Type of Mutant 14 I12N K260N + random mutant 18 L13P A130P, R181Q + random mutant 32 I12N Q121P + random mutant 41 I5M, I16T NA + random mutant 43 I16N T134S, K164I, Q186L, N210S + random mutant 10PP I9N, K27R NA.sup.d + random mutant 18PP L13P NA + random mutant LZ12.V I12V Deletion of aa 1-112 + PCR; random 215.T NA Deletion of aa 1-112; A215T + PCR; random 255.F NA Deletion of aa 1-112; S215F - PCR; site-directed FL194W NA C194W + PCR; site-directed 194.W NA Deletion of aa 1-112; C194W + PCR; site-directed 194.S NA Deletion of aa 1-112; C194S ND.sup.e PCR; site-directed 194.A NA Deletion of aa 1-112; C194A ND.sup.e PCR; site-directed 194.D NA Deletion of aa 1-112; C194D ND.sup.e PCR; site-directed 194.K NA Deletion of aa 1-112; C194K ND.sup.e PCR; site-directed .sup.aMutations are given as the residue present in the native peptide, the residue number, and the residue present in the mutein. Residue numbers for zipper domain mutations are relative to SEQ ID NO:17. .sup.bResidue numbers for mutations in the CD40L domain are relative to SEQ ID NO:12. .sup.cMutant .sup.cMutant 10PP also contained mutations in regions other than CD40L domain or the zipper domain (T-4S, D-2P, relative to SEQ ID NO:21). .sup.dNot applicable .sup.eNot done

CLAIMS:

1. An isolated CD40-L polypeptide that binds CD40, comprising a nucleic acid

encoding the CD40L polypeptide, wherein said nucleic acid hybridizes to a polynucleotide selected from the group consisting of (a)-(f), or its complement, under moderately stringent conditions (prewashing solution of 5.times.SSC, 0.5% SDS, 1.0 mM EDTA, (pH 8.0) and hybridization conditions of 50.degree. C., 5.times.SSC, overnight): (a) a polynucleotide comprising nucleotides 46 through 828 of SEQ ID NO:11; (b) a polynucleotide comprising nucleotides 184 through 828 of SEQ ID NO:11; (c) a polynucleotide comprising nucleotides 196 through 828 of SEQ ID NO:11; (d) a polynucleotide comprising nucleotides 403 through 828 of SEQ ID NO:11; (e) a polynucleotide comprising nucleotides 382 through 828 of SEQ ID NO:11; and (f) a polynucleotide comprising nucleotides 379 through 828 of SEQ ID NO:11, with the proviso that the CD40-L lacks native-pattern glycosylation.

2. An isolated CD40-L polypeptide that binds CD40, comprising a nucleic acid encoding the CD40L polypeptide, wherein said nucleic acid hybridizes to a polynucleotide selected from the group consisting of (a)-(f), or its complement, under moderately stringent conditions (prewashing solution of 5.times.SSC, 0.5% SDS, 1.0 mM EDTA, (pH 8.0) and hybridization conditions of 50.degree. C., 5.times.SSC, overnight): (a) a polynucleotide comprising nucleotides 46 through 828 of SEQ ID NO:11; (b) a polynucleotide comprising nucleotides 184 through 828 of SEQ ID NO:11; (c) a polynucleotide comprising nucleotides 196 through 828 of SEQ ID NO:11; (d) a polynucleotidecomprising nucleotides 403 through 828 of SEQ ID NO:11; (e) a polynucleotide comprising nucleotides 382 through 828 of SEQ ID NO:11; and (f) a polynucleotide comprising nucleotides 379 through 828 of SEQ ID NO:11, with the provisos that the nucleotides encoding cysteine at nucleotides 625 to 627 of SEQ ID NO: 11 are substituted with DNA encoding tryptophan and that the CD40-L lacks native-pattern glycosylation.

6. An isolated CD40-L polypeptide that binds CD40, comprising a nucleic acid encoding the CD40-L polypeptide, wherein the nucleic acid hybridizes to the complement of a nucleic acid that encodes a CD40L polypeptide selected from the group consisting of polypeptides (a)-(f), and wherein the hybridization conditions are moderately stringent (prewashing solution of 5.times.SSC, 0.5% SDS, 1.0 mM EDTA, (pH 8.0) and hybridization conditions of 50.degree. C., 5.times.SSC, overnight): (a) a polypeptide comprising amino acids 1 through 261 of SEQ ID NO:12; (b) a polypeptide comprising amino acids 47 through 261 of SEQ ID NO:12; (c) a polypeptide comprising amino acids 51 through 261 of SEQ ID NO:12; (d) a polypeptide comprising amino acids 120 through 261 of SEQ ID NO:12; (e) a polypeptide comprising amino acids 113 through 261 of SEQ ID NO:12; and (f) a polypeptide comprising amino acids 112 through 261 of SEQ ID NO:12, with the proviso that the CD40-L lacks native-pattern glycosylation.

7. An isolated CD40-L polypeptide that binds CD40, comprising a nucleic acid encoding the CD40-L polypeptide, wherein the nucleic acid hybridizes to the complement of a nucleic acid that encodes a CD40L polypeptide selected from the group consisting of polypeptides (a)-(b), and wherein the hybridization conditions are moderately stringent (prewashing solution of 5.times.SSC, 0.5% SDS, 1.0 mM EDTA, (pH 8.0) and hybridization conditions of 50.degree. C., 5.times.SSC, overnight): (a) a polypeptide comprising amino acids 1 through 261 of SEQ ID NO:12; and (b) a polypeptide comprising amino acids 1 through 261 of SEQ ID NO:12, with the provisos that the cysteine at amino acid 194 is substituted with tyrptophan and that the CD40-L polypeptide lacks native-pattern glycosylation.

8. An isolated CD40-L polypeptide that binds CD40, comprising a nucleic acid encoding the CD40-L polypeptide, wherein the nucleic acid hybridizes to the complement of a nucleic acid that encodes a CD40L polypeptide selected from the group consisting of polypeptides (a)-(b), and wherein the hybridization conditions are moderately stringent (prewashing solution of 5.times.SSC, 0.5% SDS, 1.0 mM EDTA, (pH 8.0) and hybridization conditions of 50.degree. C., 5.times.SSC, overnight): (a) a polypeptide comprising amino acids 47 through 261 of SEQ ID NO:12; and (b) a polypeptide comprising amino acids 47 through 261 of SEQ ID NO:12,

with the provisos that the cysteine at amino acid 194 is substituted with tyrptophan tyrptophan and that the CD40-L polypeptide lacks native-pattern glycosylation.

9. An isolated CD40-L polypeptide that binds CD40, comprising a nucleic acid encoding the CD40-L polypeptide, wherein the nucleic acid hybridizes to the complement of a nucleic acid that encodes a CD40L polypeptide selected from the group consisting of polypeptides (a)-(b), and wherein the hybridization conditions are moderately stringent (prewashing solution of 5.times.SSC, 0.5% SDS, 1.0 mM EDTA, (pH 8.0) and hybridization conditions of 50.degree. C., 5.times.SSC, overnight): (a) a polypeptide comprising amino acids 51 through 261 of SEQ ID NO:12; and (b) a polypeptide comprising amino acids 51 through 261 of SEQ ID NO:12, with the provisos that the cysteine at amino acid 194 is substituted with tyrptophan and that the CD40-L polypeptide lacks native-pattern glycosylation.

10. An isolated CD40-L polypeptide that binds CD40, comprising a nucleic acid encoding the CD40-L polypeptide, wherein the nucleic acid hybridizes to the complement of a nucleic acid that encodes a CD40L polypeptide selected from the group consisting of polypeptides (a)-(b), and wherein the hybridization conditions are moderately stringent (prewashing solution of 5.times.SSC, 0.5% SDS, 1.0 mM EDTA, (pH 8.0) and hybridization conditions of 50.degree. C., 5.times.SSC, overnight): (a) a polypeptide comprising amino acids 120 through 261 of SEQ ID NO:12; and (b) a polypeptide comprising amino acids 120 through 261 of SEQ ID NO:12, with the provisos that the cysteine at amino acid 194 is substituted with tyrptophan and that the CD40-L polypeptide lacks native-pattern glycosylation.

11. An isolated CD40-L polypeptide that binds CD40, comprising a nucleic acid encoding the CD40-L polypeptide, wherein the nucleic acid hybridizes to the complement of a nucleic acid that encodes a CD40L polypeptide selected from the group consisting of polypeptides (a)-(b), and wherein the hybridization conditions are moderately stringent (prewashing solution of 5.times.SSC, 0.5% SDS, 1.0 mM EDTA, (pH 8.0) and hybridization conditions of 50.degree. C., 5.times.SSC, overnight): (a) a polypeptide comprising amino acids 113 through 261 of SEQ ID NO:12; and (b) a polypeptide comprising amino acids 113 through 261 of SEQ ID NO:12, with the provisos that the cysteine at amino acid 194 is substituted with tyrptophan and that the CD40-L polypeptide lacks native-pattern glycosylation.

12. An isolated CD40-L polypeptide that binds CD40, comprising a nucleic acid encoding the CD40-L polypeptide, wherein the nucleic acid hybridizes to the complement of a nucleic acid that encodes a CD40L polypeptide selected from the group consisting of polypeptides (a)-(b), and wherein the hybridization conditions are moderately stringent (prewashing solution of 5.times.SSC, 0.5% SDS, 1.0 mM EDTA, (pH 8.0) and hybridization conditions of 50.degree. C., 5.times.SSC, overnight): (a) a polypeptide comprising amino acids 112 through 261 of SEQ ID NO:12; and (b) a polypeptide comprising amino acids 112 through 261 of SEQ ID NO:12, with the provisos that the cysteine at amino acid 194 is substituted with tyrptophan and that the CD40-L polypeptide lacks native-pattern glycosylation.

DOCUMENT-IDENTIFIER: US 20040048803 A1
TITLE: Compounds and methods for the modulation of CD154

Detail Description Paragraph:
[0184] The cleavage of CD40L into sCD40L was revealed with immunoblotting of different forms of CD40L in the platelet lysates. Cleavage of the full length CD40L (32 kD) yields the 18 kD soluble from (sCD40L). 500 .mu.l of Washed platelets were gently mixed with TRAP (5 .mu.M) to initiate the platelet activation. Metalloproteinase inhibitors GM6001 (50 .mu.M, Calbiochem) or DMSO were added to washed platelets, and incubated at 37.degree. C. with or without rocking for 30 minutes. Platelets aggregated when rocked in the presence of TRAP, but were activated but not aggregated without rocking. Platelets were removed from suspension by centrifugation for 10 minutes at 15,000.times.g, 4.degree. C. and the resulting platelet pellets were dissolved in the loading buffer. The proteins in the platelet lysates were resolved with SDS-PAGE, transferred onto nitrocellulose membrane, and blotted with anti-CD40L antibody (MK13A4, Alexis).

Detail Description Paragraph:
[0185] FIG. 4 shows that in these experiments, TRAP induced the cleavage of CD40L to sCD40L both in the sample of activated platelets and in the platelets aggregation (agg). GM6001 (GM) inhibited the cleavage of the full length CD40L to the 18 kD soluble from. Same results were obtained with another metalloprotease inhibitor TAPI-1 (Peptide International, Inc.) A separated example showed that a control inactive compound GM1012 (Calbiochem) did not affect the cleavage. Therefore, the hydrolysis of CD40L was attributed to the function of metalloproteases in the platelets.